CXCR6 Promotes Atherosclerosis by Supporting T-Cell Homing, Interferon-γ Production, and Macrophage Accumulation in the Aortic Wall

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Background—T lymphocytes are thought to be important in atherosclerosis, but very little is known about the mechanisms of lymphocyte recruitment into atherosclerosis-prone aortas. In this study we tested the hypothesis that CXCR6, a chemokine receptor that is expressed on a subset of $CD4^+$ T helper 1 cells and natural killer T cells, is involved in lymphocyte homing into the aortic wall and modulates the development and progression of atherosclerosis.

Methods and Results—To investigate the role of CXCR6 in the development and progression of atherosclerosis, we bred CXCR6-deficient ($CXCR6^{GFP/GFP}$) mice with apolipoprotein E–deficient ($ApoE^{-/-}$) mice. We found that $CXCR6^{GFP/GFP}$/$ApoE^{-/-}$ mice fed a Western diet for 17 weeks or a chow diet for 56 weeks had decreased atherosclerosis compared with $ApoE^{-/-}$ controls. Flow cytometry analysis of the aortas from $CXCR6^{GFP/GFP}$/$ApoE^{-/-}$ mice showed that the reduction of atherosclerosis was accompanied by a decreased percentage of $CXCR6^+$ T cells within the aortas. Short-term homing experiments demonstrated that CXCR6 is involved in the recruitment of $CXCR6^+$ leukocytes into the atherosclerosis-prone aortic wall. The reduced percentage of $CXCR6^+$ T cells within the aortas resulted in significantly diminished production of interferon-γ and reduction of $CD11b^+$/$CD68^+$ macrophages in the aorta.

Conclusions—These data provide evidence for a proatherosclerotic role of CXCR6. Absence of CXCR6 alters the recruitment of $CXCR6^+$ leukocytes and modulates the local immune response within the aortic wall. (***Circulation*. 2007; 116:000-000.**)

Key Words: atherosclerosis ■ immune system ■ leukocytes ■ lymphocytes ■ vessels

Atherosclerotic lesions are characterized by lipid accumulation, cell death, fibrosis, and chronic vascular inflammation.[1] The body of evidence is growing that T and B lymphocytes, dendritic cells, and macrophages reside within the noninflamed aortic wall, and a significant influx of macrophages and T cells correlates with the development and progression of atherosclerosis.[2–4] The molecular mechanisms, kinetics of trafficking, and retention of different types of immune cells within the noninflamed and atherosclerosis-prone aortic wall are not well defined. Most of the focus to date has been on the ability of monocytes to migrate to the atherosclerotic wall.[5] P-selectin, vascular cell adhesion molecule-1 (VCAM-1), P-selectin glycoprotein ligand-1 (PSGL-1), and $\alpha_4\beta_1$ integrin (VLA-4) are all involved in monocyte recruitment into atherosclerotic plaques,[6] and the chemokines CCL5 (regulated on activation, normal T cell expressed and secreted [RANTES]) and CXCL1 (keratinocyte-derived chemokine [KC]) are responsible for triggering monocyte adhesion on the atherosclerotic endothelium.[7–9] Lymphocyte trafficking into normal and atherosclerotic aortas is partially L-selectin dependent,[2] but little is known about other potential molecular mechanisms of lymphocyte recruitment.

Clinical Perspective p ●●●

CXCR6 is a chemokine receptor expressed on some T helper 1 (Th1) and natural killer T (NKT) cells found in rheumatoid joints and inflamed livers.[10] CXCR6, also known as Bonzo/STRL33/TYMSTR,[11] is expressed on subpopulations of $CD4^+$ effector memory T cells,[10] on Vα 24^+ NKT cells,[12,13] a Forkhead Box $P3^+$ (Foxp3) subset ($CD69^+$/$CD45RO^+$) of regulatory T cells in the tonsils,[14] bone marrow plasma cells, and myeloma cells.[15] In mice, interstitial intraepithelial lymphocytes, NKT cells,[16] and memory subsets of $CD8^+$ and $CD4^+$ T cells express CXCR6, which is upregulated by interleukin-2 and interleukin-15 during short-term culture.[17] Recently, lipopolysaccharide-induced toll-like receptor 4–dependent CXCR6 expression has been shown in aortic smooth muscle cells.[18]

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CXCL16 is the only known ligand for CXCR6 and is expressed by dendritic cells,[16] macrophages,[19] T cells,[20] and cytokine-stimulated smooth muscle and endothelial cells.[21,22] The disintegrin and metalloproteinase ADAM10 is involved in CXCL16 cleavage from the cell membrane,[21] leading to the release of a soluble protein that functions as a chemoattractant for responsive leukocytes.

In addition to its properties as a chemokine, CXCL16 acts also as a scavenger receptor for apoptotic cells, phosphatidylserine, and oxidized low-density lipoprotein.[16,19] Expression of CXCL16 has been reported in atherosclerosis-prone vessels from apolipoprotein-E–deficient ($ApoE^{-/-}$) mice[23] and human atherosclerotic plaques.[24] Absence of CXCL16 has been shown to accelerate atherosclerosis, enhance macrophage recruitment, and elevate mRNA levels for CCL2 (monocyte chemotactic protein-1) and tumor necrosis factor-α.[25] Because the absence of CXCL16 results in the loss of scavenger function as well as chemotactic activity, inactivating the CXCL16 gene does not separate the roles of these 2 functions in atherosclerosis.

To investigate the involvement of the chemokine receptor CXCR6 in atherosclerosis, we crossed CXCR6-GFP knockin mice[17] to atherosclerosis-prone $ApoE^{-/-}$ mice. Because Th1 cells are thought to participate in the development of atherosclerosis,[26] we hypothesized that CXCR6, a receptor highly expressed on these cells, might be relevant for their trafficking into atherosclerotic vessel wall. In mice carrying 1 functional copy of CXCR6 ($CXCR6^{GFP/+}/ApoE^{-/-}$) and mice that lack functional CXCR6 ($CXCR6^{GFP/GFP}/ApoE^{-/-}$), GFP-expressing cells were tracked by flow cytometry. Lesion size and composition were assessed by en face staining and aortic root histology. Homing of $CXCR6^{+}$ leukocytes was determined by flow cytometry. Functional consequences of CXCR6 cell recruitment were assessed as cytokine production and macrophage infiltration.

Methods

Mice

$CXCR6^{GFP/+}$ and $CXCR6^{GFP/GFP}$ mice (backcrossed onto C57BL/6 >8 generations, 98.18% of C57BL/6 [microsatellite marker screening, Charles River Lab]) were a kind gift of Dr Littman (Howard Hughes Medical Institute, New York University). $ApoE^{-/-}$ mice (C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, Maine) and crossed with CXCR6 knockin mice to obtain $CXCR6^{GFP/+}/ApoE^{-/-}$ and $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice. Mice were bred and maintained under specific pathogen-free conditions in the barrier of the University of Virginia, Charlottesville. Mice appeared healthy and were used at ages 8 to 60 weeks. All animal experiments were approved by the institutional Animal Care and Use Committee.

Preparation of Mouse Aortas and Quantification of Atherosclerosis

The aortas of mice were collected and stained with oil red O.[27] Images were scanned, and the percent surface areas occupied by lesions were determined with Image-ProPlus (Media Cybernetics). From the point of the appearance of aortic valve leaflets, sequential 5-μm-thick sections were cut, and 4 sections >300 μm were collected and analyzed by Movat staining.[28]

Flow Cytometry Analysis of Immune Cells Within Murine Aorta

Anesthetized mice were perfused by cardiac puncture with PBS containing 20 U/mL of heparin. Harvested aortas were digested with 125 U/mL collagenase type XI, 60 U/mL hyaluronidase type I-s, 60 U/mL DNAse1, and 450 U/mL collagenase type I (all enzymes, Sigma, St Louis, Mo) in PBS containing 20 mmol/L HEPES at 37°C for 1 hour.[2] A cell suspension was obtained by mashing the aorta through a 70-μm strainer. Cells were incubated with antibodies for 20 minutes at 4°C, washed twice, and incubated with secondary antibodies for an additional 20 minutes. After they were washed, immunofluorescence was detected by flow cytometry (FACSCalibur, BD Immunocytometry Systems, San Jose, Calif) or CyanADP (Dako, Colo), and data were analyzed with the use of FlowJO (Tree Star Inc, Ashland, Ore) software. PE-Texas Red-CD45, APC-Cy7 or Pe-Cy5-CD3, APC-TCRβ, PE-I-A^b, NK1.1-APC, CD62L-APC, Ly6C-biotin antibodies, and streptavidin-Per-CP, APC-Cy7 were from BD Biosciences, San Jose, Calif; CD115-PE from eBioscience (San Diego, Calif); Pacific Blue-CD11b and streptavidin-PE-Texas Red from Caltag (Burlingame, Calif); and FITC-CD68 from Serotec, Raleigh, NC. Biotin-conjugated anti-rat and anti-hamster antibodies were from Vector Laboratories, Inc (Burlingame, Calif). CCR2 antibody was kindly provided by M. Mack (University of Regensburg, Germany). In some experiments, the aortas from 2 to 3 mice were pooled and analyzed.

In Vivo Trafficking Experiments

Splenocytes from $CXCR6^{GFP/+}/ApoE^{-/-}$ mice were labeled with 7 μmol 5- (and 6)-([{4-chloromethyl}benzoyl]amino) tetramethylrhodamine (CMTMR) (Molecular Probes, Eugene, Ore) in RPMI at 37°C for 20 minutes. In some experiments, the cell suspension from $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice was labeled instead. Cells from $CXCR6^{GFP/+}/ApoE^{-/-}$ and $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice were mixed 1:1, and 35×10^6 labeled cells of each population were injected intravenously into recipient mice. At 24 hours, aortas, spleens, and inguinal, axillary, and cervical lymph nodes (peripheral lymph nodes [PLN]) were harvested. Cell suspensions were stained for CD45 and analyzed for CMTMR and GFP expression by flow cytometry. Homing of $CXCR6^{GFP/GFP}/ApoE^{-/-}$ lymphocytes was expressed relative to $CXCR6^{GFP/+}/ApoE^{-/-}$ cells (=100%).

In separate experiments, splenocytes from $CXCR6^{GFP/+}/ApoE^{-/-}$ mice (8 weeks old) were labeled with CMTMR and injected into atherosclerotic 30-week-old $ApoE^{-/-}$ or C57BL/6 recipient mice. PLN were collected 3 or 5 days later, and $CMTMR^{+}CD3^{+}$ cells were analyzed for GFP expression.

Detection of Cytokine Production Within the Aortic Wall

Dissected aortas were chopped into small pieces and placed in 100 μL of PBS at 37°C. After 10 minutes, supernatants were collected and cytokines were detected according to the BD Cytometric Bead Array protocol.

Immunohistochemistry

Paraformaldehyde-fixed cryosections of aortic root from $ApoE^{-/-}$ mice were incubated with a monoclonal antibody to CXCR6 (R&D, Minneapolis, Minn), antibody to CXCL16 (R&D), or respective isotype controls. Cell type–specific expression of the cryosections was characterized by costaining for Moma-2 (Serotec, Raleigh, NC) and α-smooth muscle actin (Dako, Hamburg). Next, secondary antibodies with FITC and TRITC-coupled reagents (Sigma-Aldrich, St Louis, Mo) were used. Paraformaldehyde-fixed paraffin sections of human carotid endarterectomy specimens were incubated with a monoclonal antibody to CXCR6 or CXCL16 followed by FITC-coupled secondary antibodies and costained for F4/80 (Serotec), α-smooth muscle actin (Dako, Hamburg), or CD3 (Serotec) with the use of TRITC-coupled reagents. Nuclei were counterstained with DAPI. Paraformaldehyde-fixed paraffin sections of murine aortic root were stained with Movat pentachrome[28] or with monoclonal antibody to Mac-2 (Cedarlane Laboratory, Limited) and hematoxylin.

Comparison of CXCR$^{GFP/GFP}$/ApoE$^{-/-}$ and ApoE$^{-/-}$ Mice

Genotype	Diet	Weight, g	Cholesterol, mg/dL	HDL, mg/dL	LDL, mg/dL	Triglycerides, mg/dL	n
ApoE$^{-/-}$	Chow	27.3±0.7	415±33	22±1	370±34	136±15	9
CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$	Chow	30.2±1.2	500±46	22±1	456±43	129±24	10

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein.

Statistical Analysis

Data are represented as mean±SE. Comparisons were made with the use of the unpaired Student test or Mann-Whitney test as appropriate.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

CXCR6 Deficiency Diminishes the Development of Atherosclerosis in CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ Mice

At 54 to 56 weeks of age, CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and ApoE$^{-/-}$ mice on a chow diet had comparable body weight and levels of total plasma cholesterol, high-density lipopro-

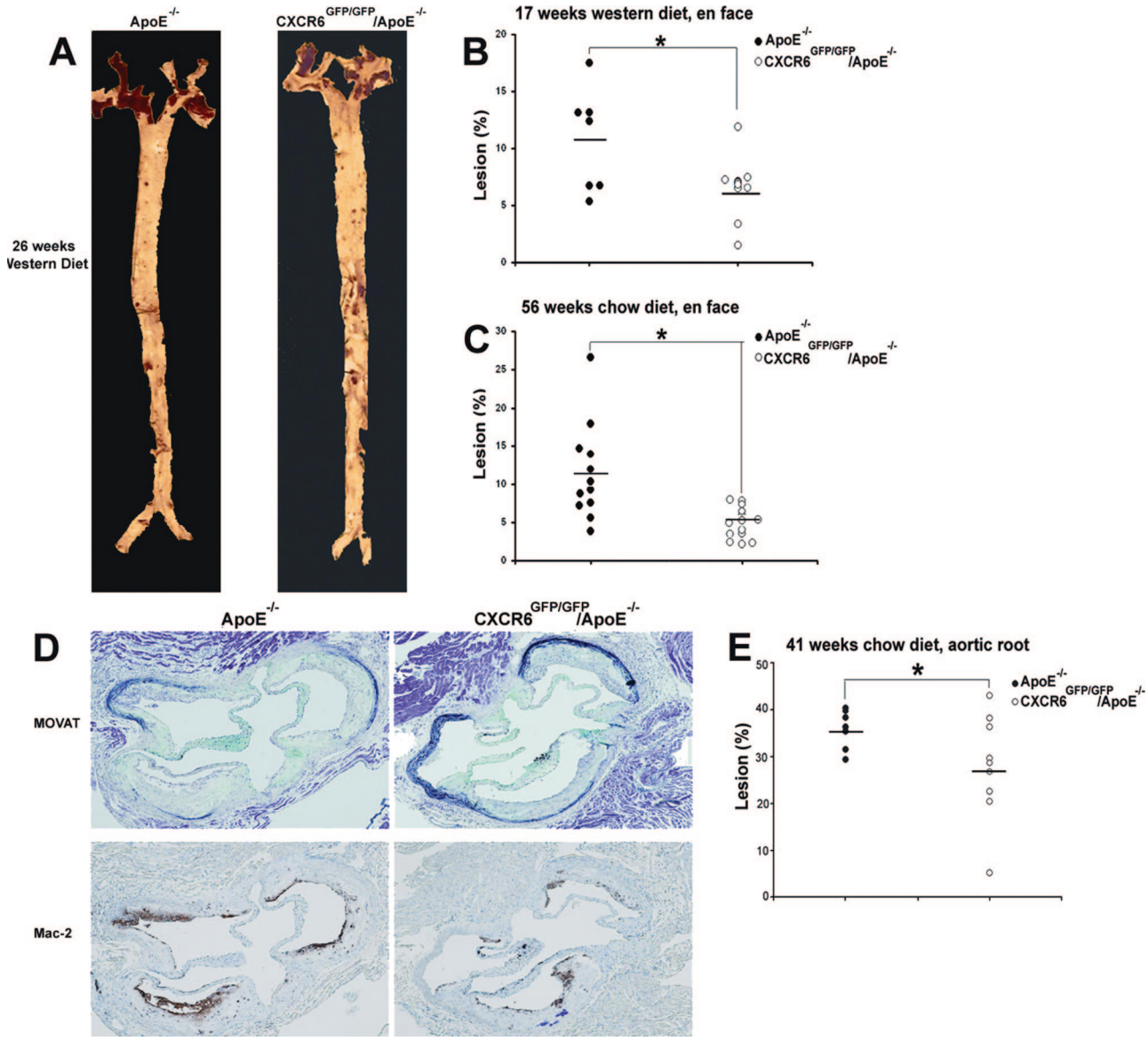


Figure 1. Absence of CXCR6 significantly reduces atherosclerotic plaque formation in CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice. A, Representative en face oil red O staining of aortas from 25- to 28-week-old CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and ApoE$^{-/-}$ mice (17 weeks of Western diet). B, Lesion sizes (% of whole aorta). Each symbol represents 1 animal, and horizontal bars represent means (for B to E). C, Lesion sizes in 56-week-old CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and ApoE$^{-/-}$ mice on a chow diet. D, Representative Movat and Mac-2 staining of the aortic root from 41-week-old CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and ApoE$^{-/-}$ mice on a chow diet. E, Lesion size (% of lumen area) within the aortic root from 41-week-old mice on a chow diet. *$P<0.05$.

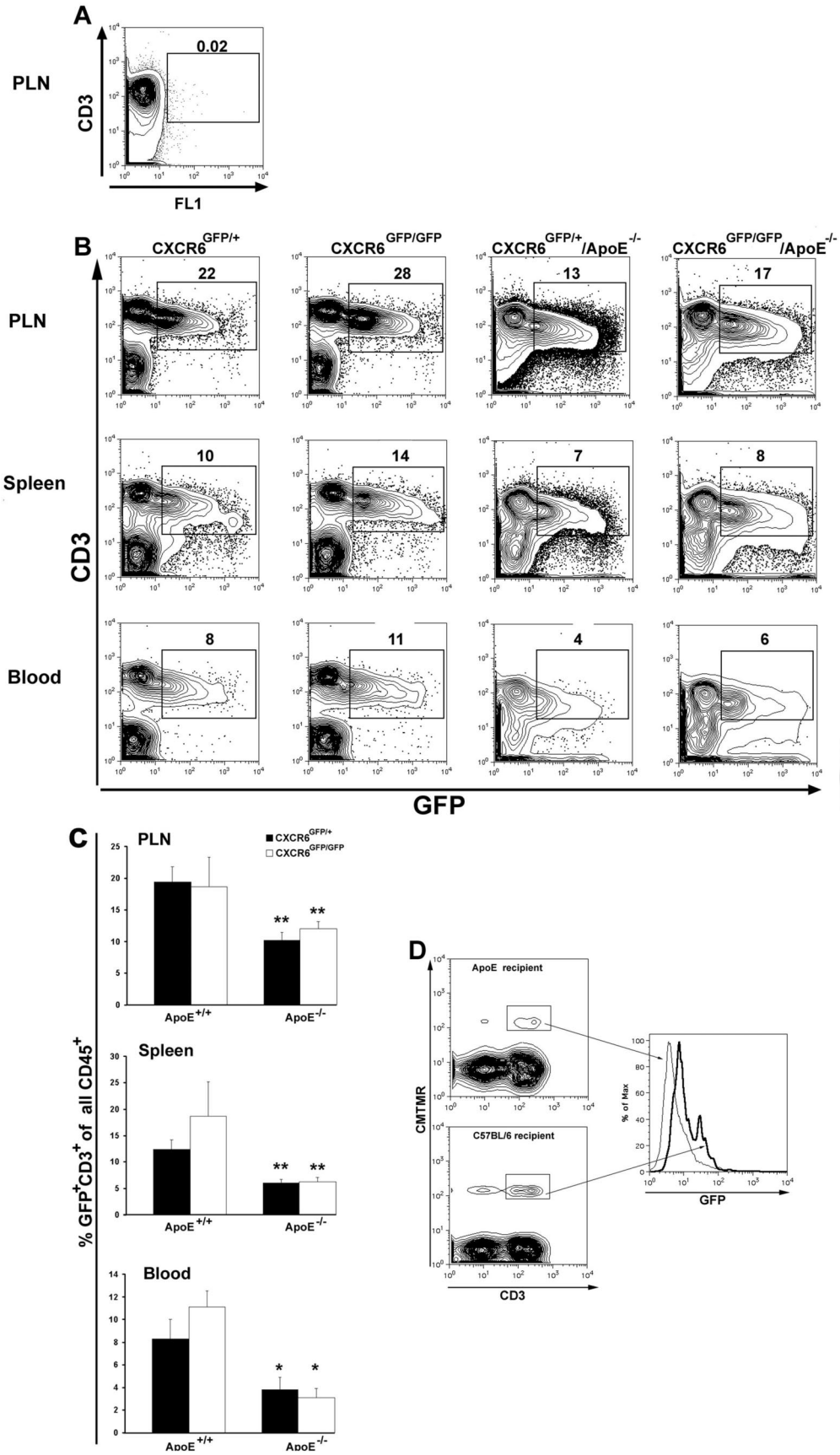


Figure 2. Deletion of ApoE reduces CXCR6-expressing CD3$^+$ cells in secondary lymphoid organs. A, Cell suspension from PLN of ApoE$^{-/-}$ mice was stained with anti-CD3 to set the gate for GFP. B, GFP expression on CD3$^+$ leukocytes in CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$

(*Continued*)

teins, low-density lipoproteins, and triglycerides (Table). Other groups of mice were fed a Western diet starting at 8 to 11 weeks for 17 weeks. This caused further elevation of total cholesterol levels but no significant difference in body weight (data not shown).

In mice placed on a Western diet for 17 weeks, CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice developed 39.5% smaller lesions throughout the aortas in comparison to those in ApoE$^{-/-}$ mice (Figure 1A and 1B). At 56 weeks on a chow diet, the average lesion size was 55% smaller in CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice than in ApoE$^{-/-}$ mice (lesions: 5.0±0.5% and 11.3±1.9%, respectively; Figure 1C). The percentage of plaque area within the aortic roots of 41-week-old mice fed a chow diet was decreased by 22% in CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice compared with ApoE$^{-/-}$ mice (Figure 1D and 1E). We also found decreased macrophage content within the aortic root of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice (Figure 1D). Partial deficiency of CXCR6 in CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice had no effect on disease progression because the average lesion size in CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice on a Western diet was not different from that in ApoE$^{-/-}$ mice (en face lesions: 13.5±1.7%). Taken together, these results suggest that CXCR6 is proatherogenic.

Reduced Number of CXCR6-Expressing T Lymphocytes in CXCR6-Knockin Mice on ApoE$^{-/-}$ Background

To investigate the influence of an atherosclerotic environment on CXCR6 expression, we compared GFP expression in PLN, spleen, and blood of CXCR6$^{GFP/GFP}$ and CXCR6$^{GFP/+}$ mice with CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice (Figure 2). To draw a gate for GFP-positive cells, C57BL/6 leukocytes (no GFP expression) and fluorescence-minus-one controls were used (Figure 2A). The gate for CD3 and other antibody staining was set up according to isotype and appropriate fluorescence-minus-one controls. Most GFP$^+$ cells analyzed in the organs of CXCR6^{GFP} mice as well as CXCR6^{GFP}/ApoE$^{-/-}$ mice were CD3$^+$ or CD3^{interm} lymphocytes with a broad range of GFP expression (Figure 2B). The percentage of GFP$^+$ cells was comparable in the PLN, spleen, and blood between CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice, but the intensity of GFP and the fraction of GFP$^+$ cells were significantly reduced compared with the same organs of CXCR6$^{GFP/GFP}$ or CXCR6$^{GFP/+}$ mice (Figure 2B and 2C). To address whether the inflammatory environment of ApoE$^{-/-}$ mice leads to CXCR6 downregulation, we performed adoptive transfer of CMTMR-labeled CXCR6$^{GFP/+}$/ApoE$^{-/-}$ splenocytes into either C57BL/6 mice or ApoE$^{-/-}$ mice and collected PLN from the recipients 3 or 5 days later. Adoptively transferred T cells (CMTMR$^+$/CD3$^+$) recovered from ApoE$^{-/-}$ recipient mice expressed lower levels of GFP compared with C57BL/6 recipient mice (Figure 2D). Thus, the expression of CXCR6 is downregulated under the atherosclerotic conditions in ApoE$^{-/-}$ mice.

Decreased Accumulation of CXCR6$^+$ Cells Within the Aorta of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ Mice

We hypothesized that the absence of CXCR6 might alter the immune cell composition of aortas. Because no reduction in the size of aortic plaque burden was detected in heterozygous CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice compared with ApoE$^{-/-}$ mice, heterozygous CXCR6$^{GFP/+}$/ApoE$^{-/-}$ knockin mice served as a control for CXCR6-deficient CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice. These flow cytometry data reflect the distribution of immune cells within the aortic wall including atherosclerotic plaques and the surrounding adventitia. Consistent with previous findings, aortas contained CD45$^+$ leukocytes (Figure 3A). The total number of CD45$^+$ leukocytes (Figure 3A) was similar in aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice ($0.68\pm0.11\times10^6$ and $0.65\pm0.16\times10^6$ per aorta, respectively). GFP$^+$ leukocytes were found in the aortas of both heterozygous and CXCR6-deficient knockin mice (Figure 3A). However, the percentage of GFP$^+$ leukocytes was significantly decreased in the aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice compared with the CXCR6$^{GFP/+}$/ApoE$^{-/-}$ controls (Figure 3A; $P<0.01$). Most GFP$^+$ cells within the aortas of CXCR6$^{GFP/+}$/ApoE$^{-/-}$ animals were CD3$^+$ or CD3^{inter} lymphocytes (Figure 3A). Flow cytometry analysis also demonstrated small populations of CD3^{inter}/NK1.1$^+$ NKT-GFP$^+$ cells at similar abundance in the aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice (Figure 3A, 3B). Among the CD3$^+$ population in the aortas, we observed a 30% decrease in the percentage of CD3$^+$/GFP$^+$ leukocytes in the aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice compared with CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice (Figure 3A, 3B). Immunofluorescence of frozen sections of murine aortas showed colocalization of CXCR6$^+$ with CD3$^+$ T cells (Figure 3C) and with some Moma-2$^+$ macrophages (Figure 3C). No colocalization of CXCR6 with smooth muscle α-actin was present (Figure 3C). As expected, the CXCR6 ligand CXCL16 was expressed in most macrophages (Figure 3C). Confocal microscopy of murine atherosclerotic aortas confirmed colocalization of some Moma-2$^+$ cells with CXCR6 (Figure 3D). Flow cytometry analysis of the aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and CXCR6$^{+/GFP}$/ApoE$^{-/-}$ mice showed that ≈10% to 20% of CD115$^+$/CD11b$^+$ leukocytes expressed CXCR6 (Figure 3E).

Decreased Trafficking of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ Lymphocytes Into the Aortic Wall

Reduced percentages of GFP$^+$/CD3$^+$ lymphocytes within the aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice suggested that CXCR6 might play a role in lymphocyte trafficking, survival, or proliferation. To test CXCR6$^+$ T cell recruitment into the aortas, we performed short-term homing assay of CXCR6$^{GFP/+}$/ApoE$^{-/-}$ and CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ lymphocytes into the

Figure 2 (Continued). CXCR6$^{GFP/+}$/ApoE$^{-/-}$, CXCR6$^{GFP/GFP}$, and CXCR6$^{GFP/+}$ mice was determined by flow cytometry. The percentage of GFP$^+$/CD3$^+$ cells is shown. C, GFP$^+$/CD3$^+$ lymphocytes (percentage of CD45$^+$) in PLN, spleen, and blood of CXCR6$^{GFP/+}$ (black bar, n=8 and n=9 for ApoE$^{+/+}$ and ApoE$^{-/-}$ background, respectively) or CXCR6$^{GFP/GFP}$ (white bar, n=3 and n=15 for ApoE$^{+/+}$ and ApoE$^{-/-}$ background, respectively) mice. Results show mean±SE. *$P<0.05$, **$P<0.01$ compared with ApoE$^{+/+}$ group. D, CXCR6$^{GFP/+}$/ApoE$^{-/-}$ splenocytes were labeled with CMTMR and injected intravenously into C57BL/6 or ApoE$^{-/-}$ recipient mice. Five days later, PLN was collected, and CMTMR$^+$/CD3$^+$ cells were analyzed for GFP expression (n=4).

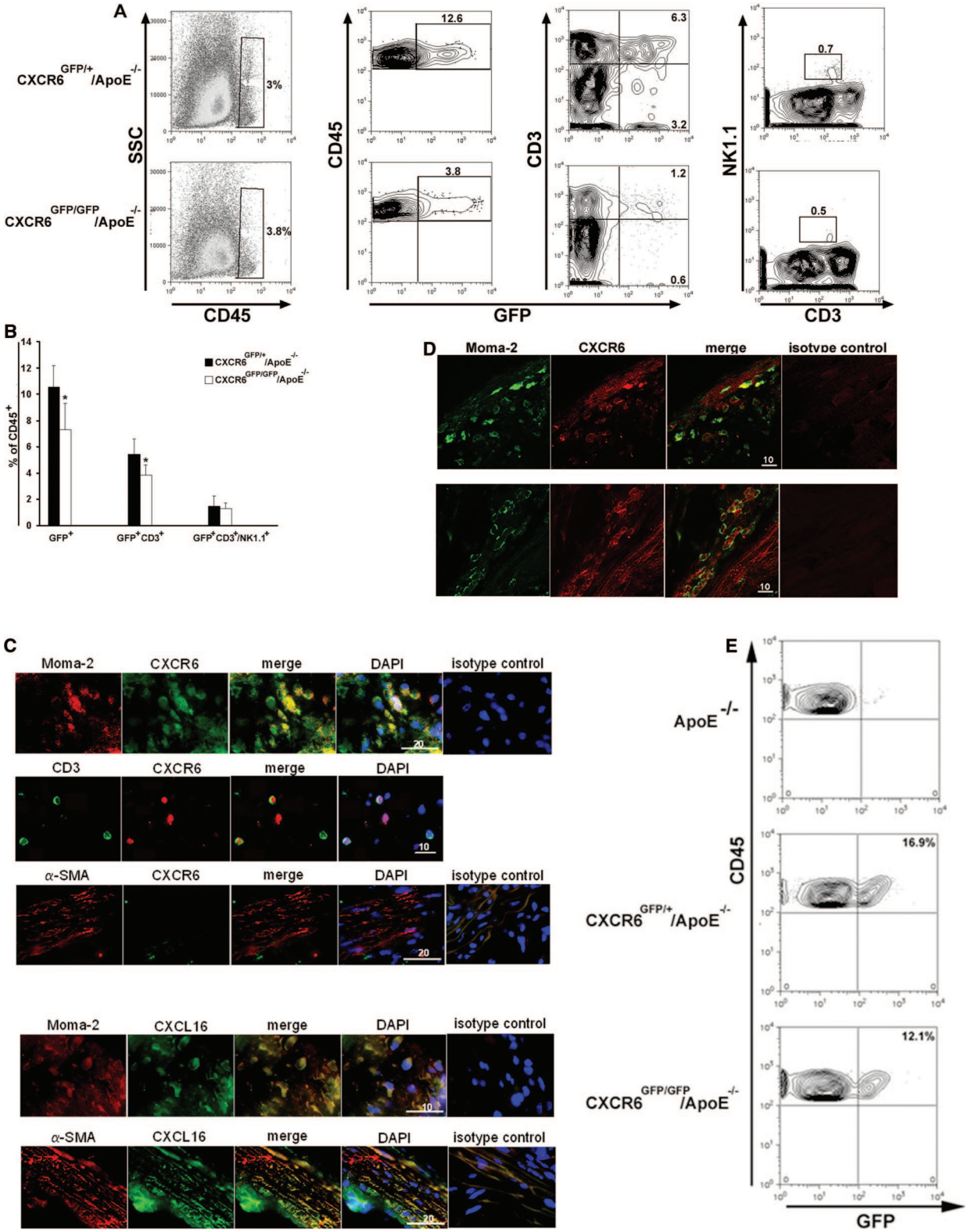
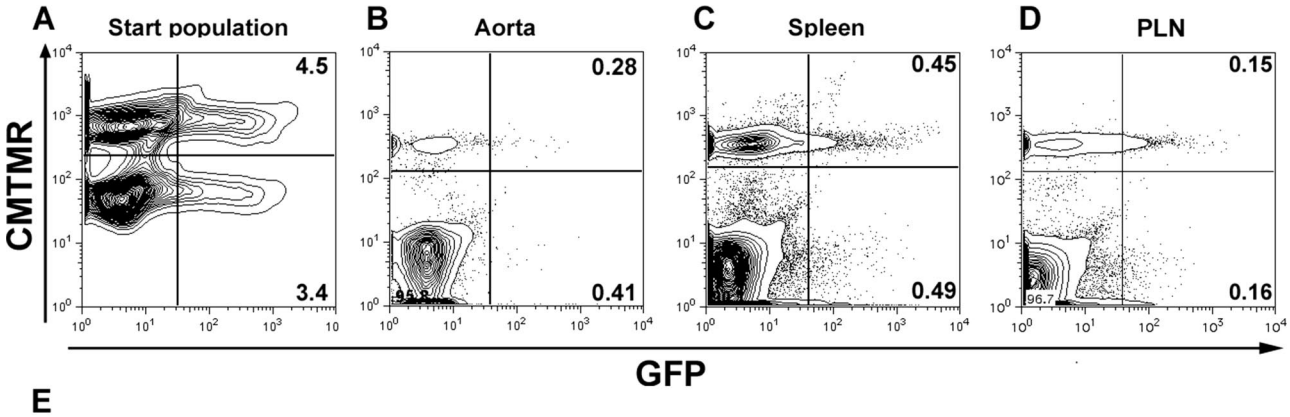


Figure 3. CXCR6 deficiency reduces the number of $CD3^+/GFP^+$ lymphocytes within the aortas of $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice. A, Aortic cell suspensions were stained for CD45, CD3, and NK1.1. Plots are gated on $CD45^+$ cells (except first column). B, Percentage of GFP^+-, GFP^+CD3^+-, $GFP^+CD3^+NK1.1^+$-expressing cells among $CD45^+$ cells from the aortas of $CXCR6^{GFP/+}/ApoE^{-/-}$ (black bars) and $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice (white bars). Results show mean±SE from 7 to 26 mice. *$P<0.05$ between $CXCR6^{GFP/GFP}/ApoE^{-/-}$ and $CXCR6^{GFP/+}/ApoE^{-/-}$ mice. C, Sections of aortic root plaques from $ApoE^{-/-}$ mice were stained for CXCR6 or CXCL16 as indicated. Colocalization with lineage markers (yellow) is shown in merged images. D, Representative confocal image of murine plaque from $ApoE^{-/-}$ mice showing colocalization of Moma-2^+ and $CXCR6^+$ cells. E, Aortic cell suspensions were stained for CD45, CD115, and CD11b, and GFP expression was analyzed by flow cytometry. Plots are gated on $CD115^+/CD11b^+$ cells (n=4 from 2 independent experiments).



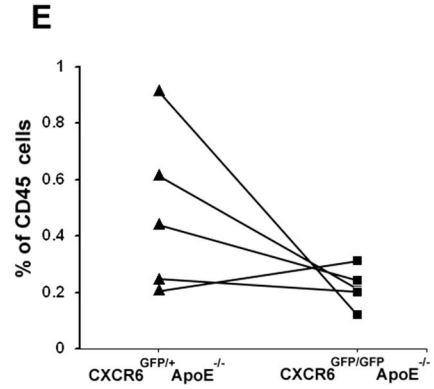


Figure 4. Absence of CXCR6 results in decreased recruitment of GFP$^+$ cells from CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice into the aortic wall. A, CXCR6$^{GFP/+}$/ApoE$^{-/-}$ or CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ splenocytes were labeled with CMTMR, mixed, and injected intravenously into recipient mice. Aortas (B), spleens (C), and PLN (D) were collected after 24 hours and analyzed by flow cytometry. Numbers in quadrants are percentage of positive cells. All plots are gated on CD45$^+$. E, Homing of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and CXCR6$^{GFP/+}$/ApoE$^{-/-}$ lymphocytes (% of CD45$^+$ cells) into the aortic wall of recipient mice (n=5 from 2 independent experiments).

aortas using CMTMR labeling (Figure 4A). CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ (GFP$^+$/CMTMR$^+$) lymphocytes showed impaired migration into the aortic wall compared with CXCR6$^{GFP/+}$/ApoE$^{-/-}$ (GFP$^+$/CMTMR$^-$) lymphocytes (0.22±0.03% versus 0.55±0.12%, respectively; n=5) (Figure 4B and 4E). Although the absence of CXCR6 significantly reduced lymphocyte homing into the aortas, it did not change lymphocyte trafficking into the spleen (0.41±0.11% versus 0.39±0.10%; n=4) and PLN (0.16±0.05% versus 0.15±0.05%; n=4) for CXCR6$^{GFP/+}$/ApoE$^{-/-}$ and CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$, respectively (Figure 4).

To provide further support for the role of CXCR6-expressing T lymphocytes in regulating the development and persistence of atherosclerosis, we evaluated the production of the Th1 cytokine interferon (IFN)-γ and the Th2 cytokine interleukin-4 within the aortas. We found a significant reduction in IFN-γ but not interleukin-4 production within the CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ aortas in comparison with the aortas of CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice (Figure 5; n=8; $P<0.05$ by t test with Bonferroni correction for multiple comparison).

Reduction of Resident Aortic Macrophages in CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ Aortas

To investigate whether CXCR6 absence might alter monocyte and macrophage distribution, monocytes in blood and macrophages in PLN and spleen (data not shown) were examined for Ly6C expression. We found no difference in the percentage of CD115$^+$/CD11b$^+$ monocytes in blood of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and ApoE$^{-/-}$ mice (8.1±1.5% and 10.8±1.5%, respectively; n=11) (Figure 6A). A distinct population existed of Ly6C^{high} blood monocytes in CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice that was not different from the subset of Ly6^{high} blood monocytes in ApoE$^{-/-}$ mice (Figure 6B and 6D). Because Ly6C^{high} monocytes are typically CCR2$^+$,[29] we analyzed monocyte subsets for CCR2 expression. We found no difference in the percentage of CD115$^+$/CCR2$^+$/Ly6C^{high,inter} monocytes in the blood of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice compared with ApoE$^{-/-}$ mice (71±5% and 64±7%, respectively; n=6). Further phenotypic characterization of Ly6C^{high} monocytes showed a similar

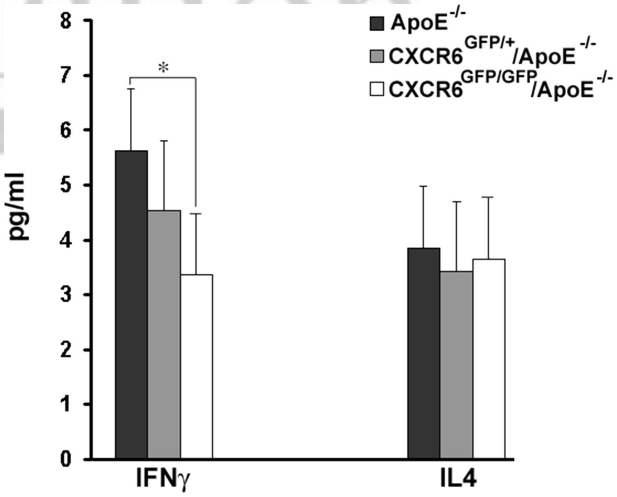


Figure 5. Reduced production of IFN-γ within the aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice. Aortas from CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ (white bars), CXCR6$^{GFP/+}$/ApoE$^{-/-}$ (gray bars), and ApoE$^{-/-}$ (black bars) were minced and incubated in PBS at 37°C for 10 minutes. Collected supernatants were analyzed for the presence of interleukin-4 and IFN-γ with the use of BD Cytometric Bead Array. Results show mean±SE from 8 to 11 mice. *$P<0.05$ between CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and ApoE$^{-/-}$ mice by Mann-Whitney test.

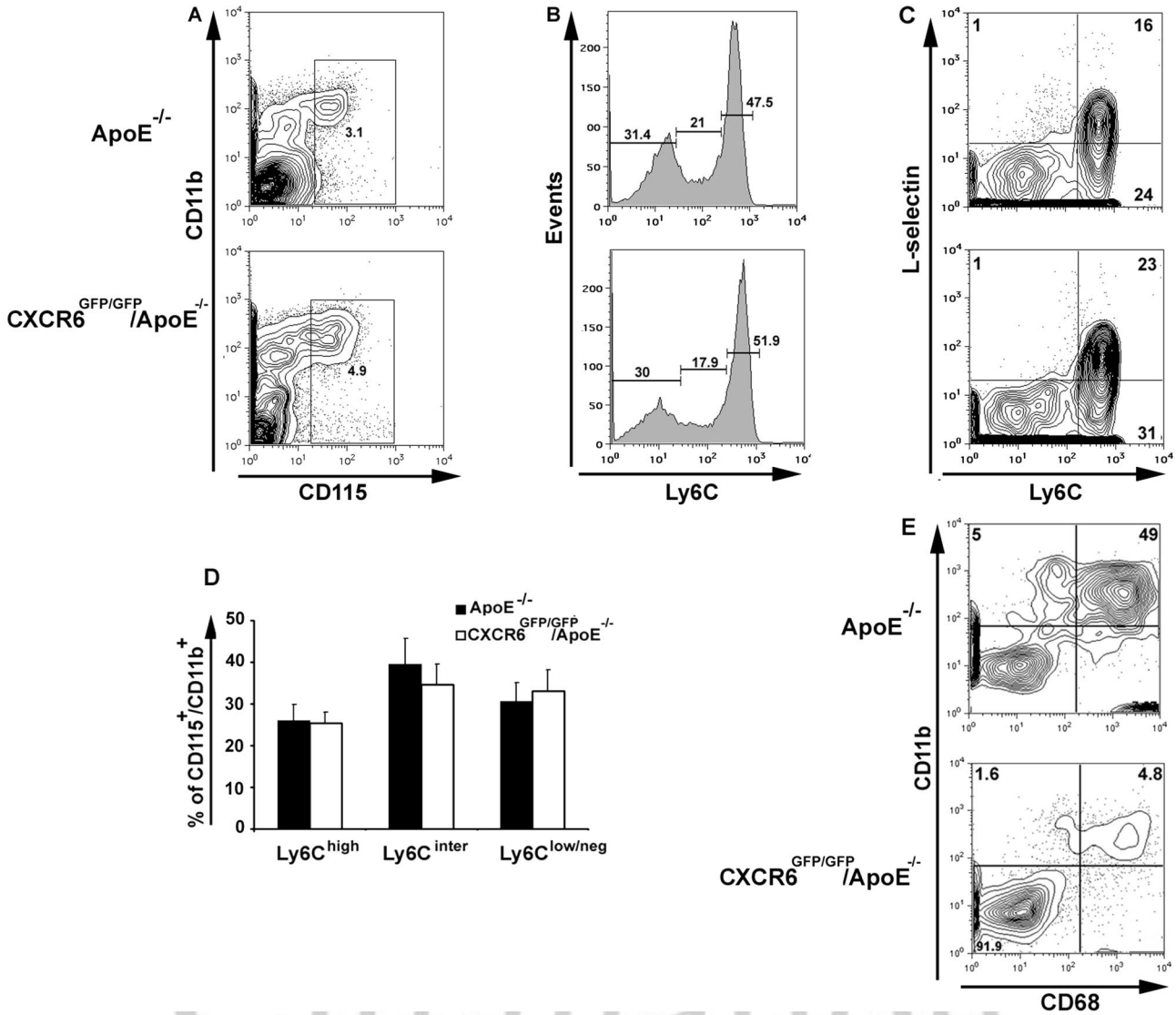


Figure 6. Absence of CXCR6 has no effect on monocyte populations in the blood but results in diminished percentage of macrophages within the aortic wall of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$. A, Monocytes were stained with CD115 and CD11b antibodies, and the percentage of monocytes in the blood was determined. B, Histograms show Ly6C expression on CD115^{+}/CD11b^{+} monocytes from blood of ApoE$^{-/-}$ and CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice. C, L-selectin expression on CD115^{+}/CD11b^{+} blood monocytes from CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice (bottom) and ApoE$^{-/-}$ mice (top). D, Average percentage of Ly6C^{high} CD115^{+}/CD11b^{+} monocytes in the blood of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ (white bar) and ApoE$^{-/-}$ (black bar) (n=11). E, Presence of CD11b^{+}/CD68^{+} macrophages within the aortas. Representative of 3 experiments (n=8). Plots are gated on CD45^{+} cells.

pattern of CD62L expression on CD115^{+}/CD11b^{+} blood monocytes of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ and ApoE$^{-/-}$ mice (Figure 6C).

Lack of CXCR6^{+} T lymphocytes may curb IFN-γ production within the aortas and thus may influence the recruitment or survival of monocytes. CD11b^{+}/CD68^{+} macrophages were reduced by 90% (Figure 6E) in the aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice compared with CXCR6$^{GFP/+}$/ApoE$^{-/-}$ mice ($P<0.0005$; n=8). The percentage of Ly6C^{high} macrophages was also reduced within the aortas of CXCR6$^{GFP/GFP}$/ApoE$^{-/-}$ mice compared with ApoE$^{-/-}$ mice (6.8±1.5% versus 15.1±2.0%, respectively; $P<0.05$; n=4).

To address the potential importance of our findings for human atherosclerosis, the expression of CXCR6 and CXCL16 mRNA was investigated in carotid endarterectomy specimens versus nondiseased arteries by quantitative reverse transcription polymerase chain reaction, demonstrating 4-fold upregulation of both the chemokine and the receptor (data not shown). Sections from carotid endarterectomies were then stained for CXCR6 and CXCL16. Atherosclerotic plaques have few T cells, and most T cells reside under the media layer within tertiary lymphoid structures.[2] Therefore, only few T cells were detected within the plaques. CXCR6 was highly expressed in some CD3^{+} T cells and F4/80^{+} macrophages but not in smooth muscle cells (Figure 7A). CXCL16 mainly colocalized with macrophages (Figure 7B). Taken together with the mouse data, this suggests that the CXCR6/CXCL16 interaction plays an important role in atherosclerosis.

Discussion

This study reveals a significant proatherogenic role of CXCR6 in atherosclerosis. Absence of CXCR6 resulted in a

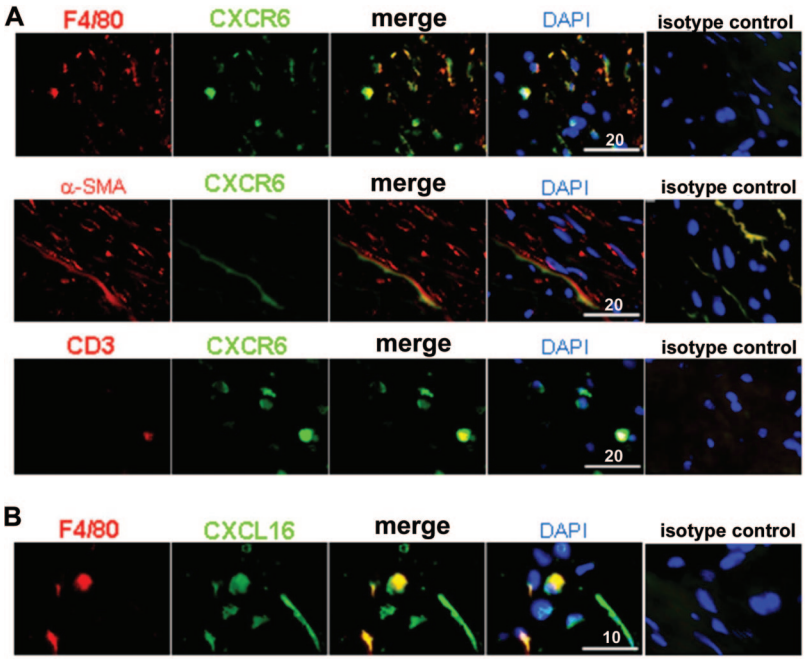


Figure 7. CXCR6 is expressed by T cells in human atherosclerotic lesions. Sections of carotid endarterectomy specimens were costained for CXCR6 (A) or CXCL16 (B) and the cell type–specific markers. Colocalization (yellow) is shown in merged images, and counterstaining was performed with DAPI (blue). Except some autofluorescence of elastic laminae, isotype control staining was negative.

≈50% reduction in lesion formation as assessed by en face staining of the whole aorta and 22% of the lesion reduction within the aortic root. The decrease in lesion size was found in 56-week-old chow-fed mice and 25-week-old mice fed a Western diet. Removing 1 functional copy of CXCR6 had no effect. Flow cytometry analysis of aortas revealed a marked reduction in aortic $GFP^+/CD3^+$ but not NKT cells in $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice that was likely due to defective trafficking into the aortic wall. The decrease in the number of $CXCR6^+$ cells was accompanied by a reduction of aortic IFN-γ production, one of the most powerful inflammatory cytokines that initiates and supports inflammation in the aorta. Even more notably, macrophages were decreased up to 90% in the aorta of $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice. Thus, CXCR6 is likely to be involved in atherosclerosis through the alteration of the homing of activated effector T cells into the aortas where those T cells produce proinflammatory cytokines, which determine macrophage recruitment.

T cells are abundant in the aortic adventitia of normal mice.[2,3,30] In atherosclerotic mice, some T cells are found in atherosclerotic plaque[31] and more in atherosclerosis-induced adventitial lymphoid structures.[2,30] In the present study we describe a T-cell population that expresses CXCR6 and resides within the aortas of mice fed with either a chow or Western diet. Interestingly, the levels of CXCR6 expression and the percentage of GFP^+/CXCR6-expressing T cells from $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice were lower than in $CXCR6^{GFP/GFP}$ mice. Adoptive transfer experiments suggest that the proinflammatory $ApoE^{-/-}$ environment reduces the levels of CXCR6 expression. CXCR6 expression is downregulated when T cells are stimulated via CD3/CD28 ligation.[32] The exact mechanism of CXCR6 downregulation remains to be determined, but one of the possible mechanisms might be downregulation secondary to T-cell receptor engagement.

Several studies have demonstrated an important role for chemokines and chemokine receptors in the recruitment of monocytes during atherosclerosis, but little is known about T-cell recruitment to the aortas. In the present study we provide evidence that CXCR6 is involved in the recruitment of Th1 cells into the aortic wall.

CXCL16 is the only known chemokine ligand for CXCR6 and plays a dual role as a chemokine and a scavenger receptor that binds oxidized low-density lipoprotein. Aslanian and Charo[25] generated $CXCL16^{-/-}$ mice and showed that CXCL16 in vivo is atheroprotective. The present data suggest that this must be due to its scavenger receptor activity. Because CXCL16 deletion results in the abolishment of both CXCL16 functions (chemotaxis and scavenging), it is impossible to determine whether the chemokine or scavenger receptor function leads to the accelerated atherosclerosis in $CXCL16^{-/-}$ mice. Shedding-resistant CXCL16 mutant mice might be useful to resolve this question. An alternative explanation for our finding is that CXCR6 may have additional ligand(s) that remain to be discovered.

The presence of NKT cells within atherosclerotic aortas and their proatherogenic role have been reported.[33] CXCR6 is broadly expressed on NKT cell populations in different organs such as the liver and spleen,[34] but the percentage of NKT cells expressing GFP in CXCR6-deficient mice is not different compared with control mice. Not only T cells but also a subset of macrophages expresses CXCR6 in atherosclerotic aortas. Further studies will be needed to determine the role of CXCR6 on macrophages. Interestingly, the absence of CXCR6 had no effect on the subpopulation of $Ly6C^{high}$ monocytes in the blood of $CXCR6^{GFP/GFP}/ApoE^{-/-}$ mice but resulted in the alteration of the number of $Ly6C^{high}$ $CD11b^+/CD68^+$ macrophages in the aorta, suggesting that the absence of CXCR6 alters the subsets of aortic macrophages residing within the aortic wall.

$CXCR6^+$ T cells have a capacity to secrete a large amount of IFN-γ on activation.[10,35] In this study we show that CXCR6-deficient aortas secrete less IFN-γ, likely because of

the decreased number of CXCR6$^+$ Th1 effector lymphocytes within the aortic wall. IFN-γ is one of the key proinflammatory cytokines that participates in atherosclerosis and increases inflammatory aspects of this disease through upregulation of proinflammatory genes.[36] The decreased level of IFN-γ in aortas of CXCR6-deficient mice might reduce macrophage recruitment. Indeed, we observed up to a 90% decrease of CD11b$^+$/CD68$^+$ MΦ within the aortas of CXCR6-deficient mice. IFN-γ also regulates the expression of CXCL10, CCL2, and CX3CL1,[36] which can further affect the recruitment of macrophages and T cells into the atherosclerotic aortic wall. It appears that CXCR6$^+$ T cells are an important source of IFN-γ that regulates the cell content of atherosclerotic lesions.

In summary, the present study establishes CXCR6 as an important chemokine receptor in the development and progression of atherosclerosis through the recruitment of CXCR6$^+$ effector T cells into atherosclerotic aortas. Our findings suggest a new pathway by which effector T cells may influence the development of atherosclerosis through the alteration of immune composition within the aortic wall.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The concept of the involvement of the immune response in the development and persistence of atherosclerosis has become firmly established; however, our knowledge of the detailed molecular mechanisms of immune cell trafficking is still incomplete. In the present study, we identified the proatherogenic role of the chemokine receptor CXCR6 in atherosclerosis. CXCR6-expressing T cells home into the aortic wall, increase the local production of the proinflammatory cytokine interferon-γ, and promote macrophage accumulation. This study suggests that interventions aimed at inhibiting CXCR6 function might be useful for treating and preventing atherosclerosis and possibly other forms of vascular disease.